12-15-2011

Discrimination and Aggregative Patterns among and between populations of Entamoeba spp.

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December 2011

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Acknowledgements

I would like to thank Dr. Avelina Espinosa and Dr. Guillermo Paz-y-Miño C. for their guidance, knowledge, and unsurpassed dedication to this project. Thank you to Dr. Tomas Sorger and Dr. Lauren Rossi for a critical reading of the manuscript, and to Caitlin McNally, Haylee Zubrycki, and Lauren Salerno for their laboratory assistance. Funding was provided by the NIH-NCRR grant # 2 P20RR16457-11, Merck/AAAS URSP grants. The study was also supported by the Roger Williams University FCAS Senior Thesis Program, and by the Roger Williams University Honors program.

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Abstract

Kin recognition facilitates the evolution of cooperation in animals, but its relevance in microorganisms and their behavior toward relatives remains unclear. The *Entamoeba* lineage constitutes an ideal model to determine the behavioral and signaling cues needed for aggregative preference. Chemical cues have been reported for *E. histolytica* but not *E. dispar*, suggesting cell-communication between con-specifics and behavioral differences with relevance for disease. *Entamoeba* varieties have been reported as 'morphologically undistinguishable'. Two strains of *E. invadens* have been isolated from different hosts (*VK-1: NS* - Varanus komodoensis and *IP1*- serpentes) but were classified within the same 'species'. Trophozoites of each strain aggregate only with members of their own variety, suggesting they are able to associate based on behavioral and chemical communication. Adaptations to different environments and horizontal gene exchange could have influenced diversification of each lineage. Measurable aggregation and behavioral cues in fluorescence micrographs of *Entamoeba* varieties suggest that these characteristics should be included in phylogenetic studies.

Background

Entamoeba spp. are unicellular pathogenic protozoa. Some 'species' and strains cause infectious diseases in vertebrates (amebiasis), and some are free living or can coexist, causing no damage to their hosts. It has recently been discovered that people showing symptoms of amebiasis are most likely infected with *Entamoeba histolytica* and that those who are asymptomatic usually host *Entamoeba dispar*. Amebiasis is one of the most common parasitic diseases occurring in humans and causes more than 100,000 deaths each year (Espinosa et al., 2009, p. 675). The parasite is primarily spread through food and water due to fecal contamination and lives in the intestines, liver, or other parts of the body. Once the parasite is located within the body, it survives by feeding off of intestinal bacteria or the liver lining.

The *Entamoeba spp*. are the focus of many phylogenetic studies. Some 'species' not only are easily isolated and cultured, but also have a rapid reproductive cycle. Behaviorists have discovered behavioral and social aspects in highly cognitive and social organisms; however, little has been explored within microbial groups. Kin discrimination refers to an individual's capability to determine a genetic relative from a distant-relative. The term "kin discrimination differs from kin recognition in that the latter term refers to cognitive processes, whereas kin discrimination describes observable behavioral patterns" (Ostrowski, 2008, p. 363). For the technical purposes of this paper, 'discrimination' will be utilized.

E. invadens-VK1-NS and *IP1* are non-pathogenic reptilian parasites currently classified as two strains within the same species. *E. invadens-VK1-NS* was isolated from komodo dragons (Varanus komodoensis); *E. invadens- IP1* was isolated from snakes (Serpentes) (Dey et al., 2009, p. 245). Both subspecies are morphologically and biochemically distinct and have adapted to their hosts' environments. *E. invadens* can exist within some reptiles as a communal organism,

but is pathogenic in a subset of hosts, in which it causes a similar type of colon pathology to that of *E. histolytica* observed in infected humans (Donalson et al., 1975, p. 808). *E. invadens* have the same basic morphology and life cycle as *E. histolytica*, forming quadric-nucleated cysts with chitinaceous, osmotically-resistant cyst walls; however, in axenic cultures, *E. invadens* encyst *in vitro* in response to changes in the carbon source or osmolarity of the growth medium (Wang, 2003).

Recent observations of the *Entamoeba* clade suggest that members of this lineage discriminate and associate with members of their own group (Espinosa, unpublished). Mehdiabadi et al. (2006) state that that "kin recognition helps cooperation to evolve in many animals, but it is uncertain whether certain microorganisms can also use it to focus altruistic behavior on relatives" (p. 881).

Trophozoites belonging to different 'species' and strains of *Entamoeba* are difficult to identify morphologically, and their phylogenetic classification could hide 'cryptic' groups. This study will focus on uncovering several cryptic varieties in the *Entamoeba* lineage using modern molecular tools. Understanding the mechanisms of discrimination is just one step in the direction of understanding the development of both amebiasis and evolutionary pathways.

Social ameba show sophisticated behavior previously thought to occur only in intellectually higher organisms (Mehdiabadi et al., 2006, p. 881). They hypothesized, "one or more recognition alleles can determine discrimination among amoebas" (p. 881). Mehdiabadi and collaborators (2009) have revealed that genetic variation impacts social interactions (p. 547).

Lizé and colleagues (2007) have studied kin recognition among *Aleochara bilineata* gyll, a parasite, and have concluded that identifying kin has a profound influence on competition (p.

1737). Signaling discrimination has been studied in other organisms but not among *Entamoeba* species.

The associative patterns within strains of *Entamoeba* are interesting from both a biological and clinical perspectives (Espinosa, unpublished). Chemotactic behavior in *Entamoeba histolytica* has been shown previously placing trophozoites in different media (Zaki et al., 2006). This publication examines the restrictive *in vivo* environment that the ameba are likely to be confronted during invasive disease. The movement of cells towards and away from conditioned media were observed and recorded. However, there is little experimentation on the behavioral signaling in *Entamoeba spp.* We hypothesize that *Entamoeba* varieties are able to discriminate and aggregate with members of their group using processes such as chemotactic signaling.

Research Methods and Procedures

Experimental Combinations of *E. invadens-VK1-NS* and *IP1* in Fresh Media

Basic observations of *E. invadens- IP1* and *E. invadens- VK1-NS* such as physical properties, mobility, and behavior were measured and examined under the microscope using photographic and video-recording tools. Descriptive observations of *Entamoeba* aggregation and distance between individuals were recorded for each strain.

Physical properties, such as size, could alter the mechanical processes of the strain and their interactions with kin. Physical properties are best measured using a micrometer. The average length and width, the individual cell surface area, the clusters' surface areas, the number of ameba in aggregates, and the mean diameter of individual cells were measured using

ImagePro® Plus 6.3 Software (Media Cybernetics, Inc., Bethesda, MD). The healthy shape of the cells is characterized. Some strains appear to be lima bean shaped while others assume a spherical shape at ideal conditions. Ideal conditioned for *E. invadens* include culture temperature at 23°C in NYU medium and Filtered Additive (Appendix B). The mutations that cause slight changes in physiology of these parasites could allude to pathogenesis.

Invitrogen Molecular Probes ® (Eugene, OR) CellTracker^{TM} CMPTX Red and Green CMFD fluorescent probes were utilized for the experimental assays. These probes are inherited by daughter cells after cell fusion and are not transferred to adjacent cells in a population. The reagents pass through the cell membranes, but once inside the cell membrane they are transformed into membrane-impermeable products. Using aldehyde fixatives the fluorescent dyes are fixed in the cells. The detailed fluorescence protocol can be found in Appendix A. The probes were used to tag two differing strains (same starting concentrations) in order to view the aggregative patterns and characteristics of the species. Table 1 demonstrates the combinatorial experiments performed with various repetitions:

Table 1: *Entamoeba invadens-IP1* and *VK1-NS* experimental combinations

Unlabeled (no dye)	Labeled (with red and green fluorescent dyes)
VK1 (alone)	VK1 (R)/VK1 (G)
IP1 (alone)	IP1 (R)/IP1 (G)
VK1/IP1	VK1 (R)/ IP1 (G)
_ _ _	VK1 (G)/ IP1 (R)

Unlabeled controls demonstrate amoeba behavior without the effects of the dye, allowing comparisons and conclusions to be drawn [Results Figure 1]. *E. invadens*-IP1 and *VK1-NS* species are tagged with reciprocal colors to show that the color dye has no effect on the overall

interactions between the varieties [Results Figure 2]. Each experimental combination was observed and analyzed at 12, 18, and 36 hours following the dying procedure.

The dying and fluorescing protocols for labeled combinations can be found in Appendix A [p. 25-26]. It was modified from a previously published protocol (Mehdiabadi et al., 2006). The Zeiss Axiovert 40 CFL Microscope (Göttingen, Germany) was used in each experiment to detect and measure fluorescence. The same viewer conducted all experiments under the same conditions to eliminate variability. Data was collected and analyzed. Physical observations were replicated more than three times.

Physical observations and characteristics of each experimental combination were assembled into quantitative data using ImagePro® Plus 6.3 Software (Media Cybernetics, Inc., Bethesda, MD).

Amebic behavior, motility and cell-to-cell interactions were observed, recorded and quantitated. These behavioral cues were measured using fluorescent microscopy. After fluoresced experiments were recorded with the software, the red- and green-tagged ameba were counted and measured. The surface area of each individual fluoresced ameba and the clusters (of the same color) of amebas were measured in microns. When the two fluorescent dyes overlapped, the camera recorded a yellow hue. The yellow overlapping areas indicated where ameba interactions occurred. The surface area of the yellow was also calculated using the ImagePro® Plus 6.3 Software (Media Cybernetics, Inc., Bethesda, MD). Videos and images were recorded to help view the aggregates and individual ameba in motion under 32x magnification at each time interval (12, 18, 36 hrs) in all experiments.

Aggregates are cells accumulated together into a dense cluster. Clusters are defined as group of more than 20 trophozoites. Aggregates were observed in all the strains in the laboratory, except *Entamoeba moshkovski* Larendo (Appendix B, Figure B1). The length and width of the aggregates were observed under 32x magnification and measured with ImagePro® Plus 6.3 Software (Media Cybernetics, Inc., Bethesda, MD) in microns. Mean distances between the two aggregates were recorded in each combination.

Conditioned Media Experiments

The interactions in the *E. invadens*-IP1 experiments greatly increased after 18 hours, so new experimental procedures were arranged using conditioned media [appendix A]. We hypothesize that a signaling molecule might be secreted into the media by the trophozoites to communicate with like-members. The accumulation of this signaling molecule could contribute to the induced cellular interactions of a growing culture of ameba. The media was conditioned for a shorter time (12 hours) and a longer time (18 hours) in flasks and extracted, isolated, and stored for future experimentation.

With the same fluorescent tagging procedure, *E. invadens-IP1* trophozoites were fluoresced red and green and combined. Instead of the previously used fresh media, the filtered conditioned media was added. The same procedure follows for data collection. Observations were taken at 0 hrs, 3 hours, and 21 hours after the ameba were tagged and combined.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Conditioned media (conditioned for 24, 48, and 72 hour time frames) collected from both *E. invadens- IP1* and *VK1-NS* were ran through a Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) using Coomaisse Brilliant Blue to stain the samples allowing

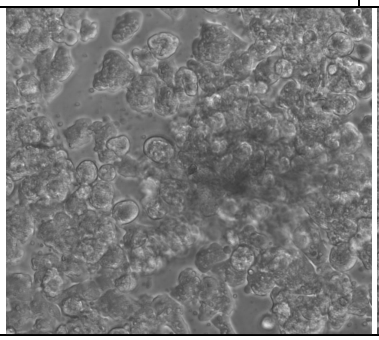
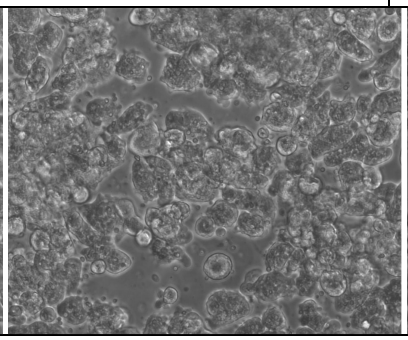
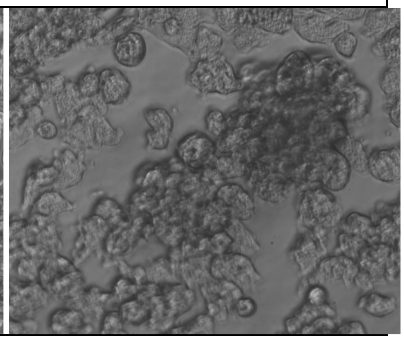
visualization of the protein fragments. Invitrogen Benchmarker molecular marker was used to calibrate the gel and determine the approximate molecular mass of the unknown protein fragments in kilodaltons by comparing the distance traveled relative to the marker. These samples were diluted 3x using purified double distilled H_2O.

Results

Experimental Combinations of *E. invadens-VK1-NS* and *IP1* in Fresh Media

The use of florescent dyes to determine physical observations of *Entamoeba* varieties has detected differences in aggregation patterns. Differing strains of *Entamoeba* prefer to aggregate with members of their own kin and maintain distance from cells of a differing variety. *E. invadens-IP1* and *E. invadens- VK1-NS* are similar strains but after analyzing quantitative values (average length, width, and surface area) it is possible to conclude that on average *E. invadens-IP1* is larger [Appendix Table B4].

Invitrogen CellTracker™ red CMTPX and Invitrogen Cell Tracer Vybrant® green CFDA SE fluorescent dyes do not have an effect on ameba health in short term experiments (approx. 40 hrs). Negative controls demonstrate ameba behavior without the effects of the dye [Figure 1].

	12 hrs	18 hrs	36 hrs
Control Negative VK			

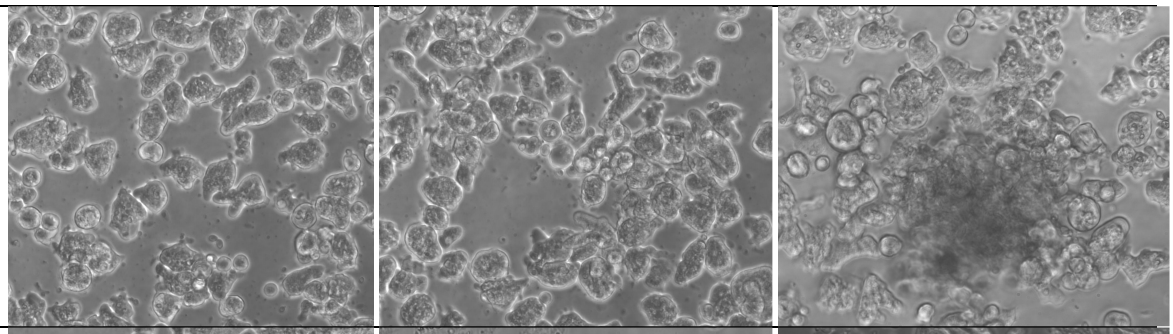
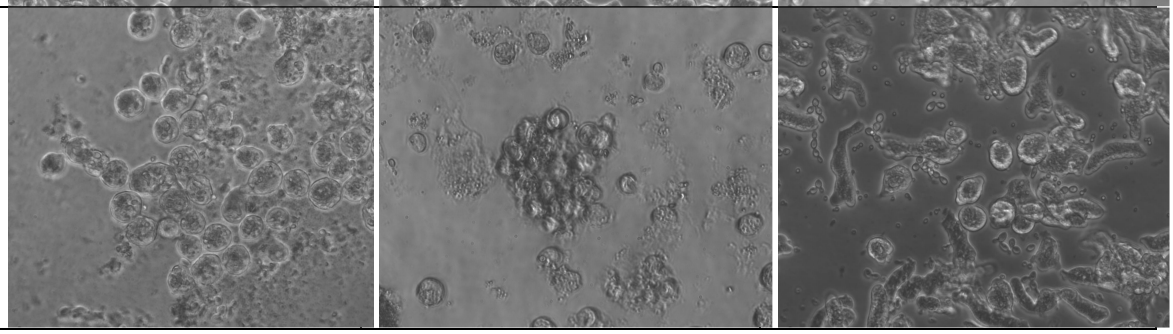
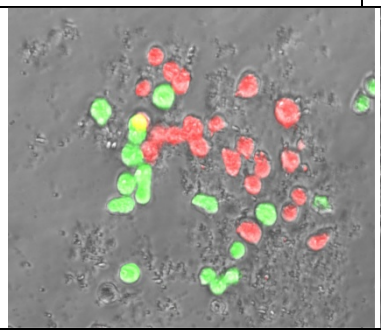
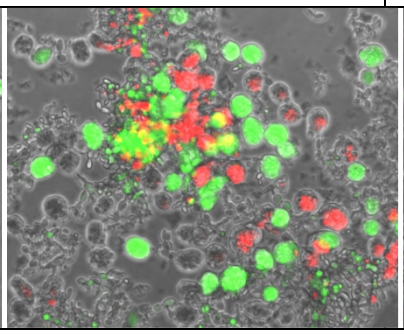
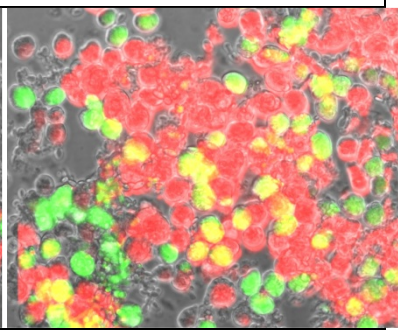
Control Negative IP1	
Control Negative VK/IP1	

Figure 1: Control combinations of *E. invadens-IP1* and *VK1-NS* in Fresh NYU media (non-conditioned, non-fluoresced) over 12, 18, and 36 hours [32x].

This reveals little difference from the labeled positive (fluorescently tagged) controls demonstrating that the dyes do not vary experimental results [Figure 2]. When comparing the unlabeled control combinations to the labeled positive controls it is seen that the molecular probes do not affect the aggregative patterns over a forty hour period either. In the reciprocal labeled *VK1-NS/IP1* combinations the red and green dye does not favor one type of strain more than the other [Figure 2].

	12 hrs	18 hrs	36 hrs
Positive VK(R)/VK(G)			

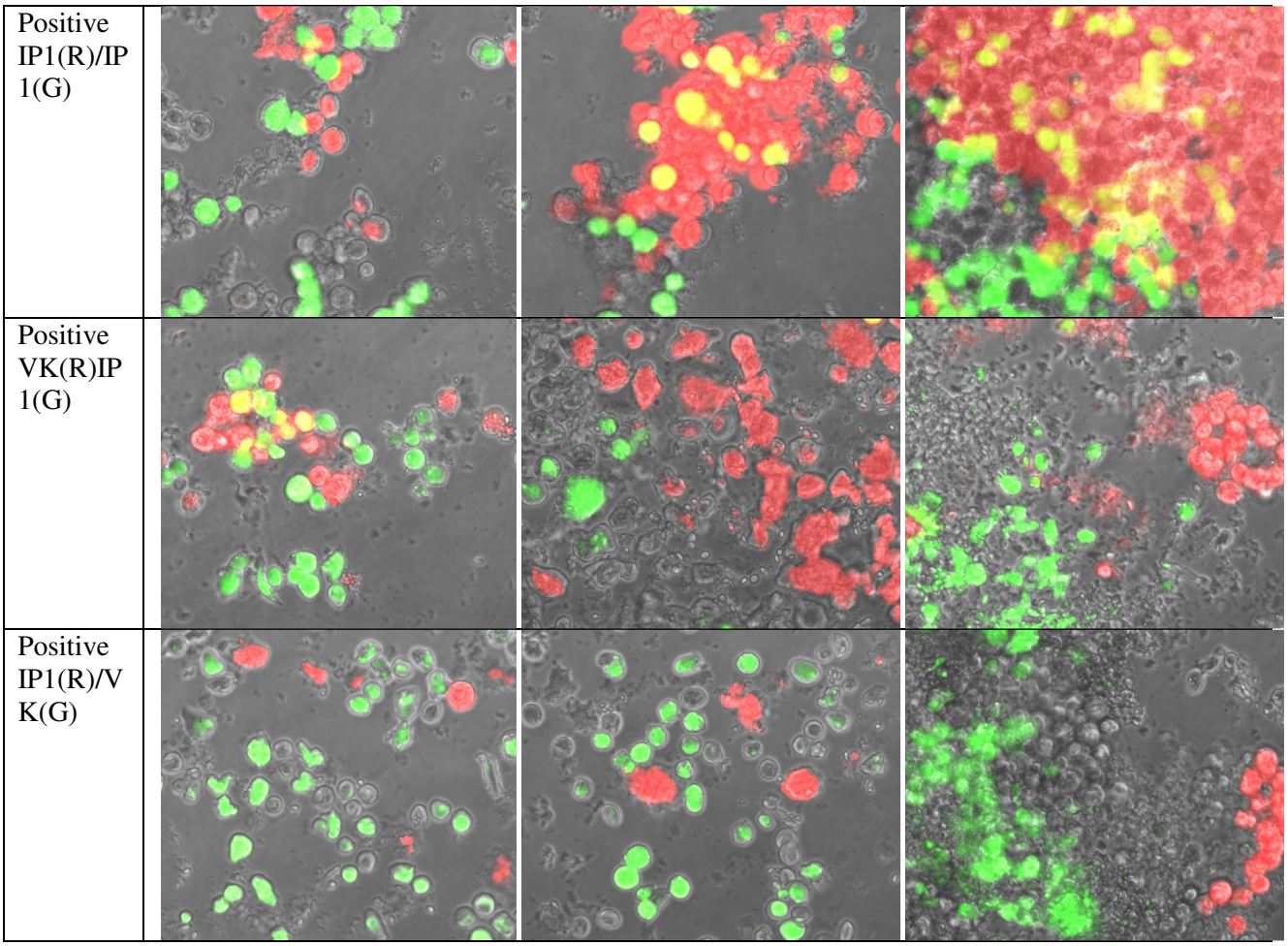


Figure 2: Fluoresced *E. invadens IP1* and *VK1-NS* in Fresh NYU media (non-conditioned) over 12, 18, and 36 hours. [32x]

When similar varieties of *E. invadens* are combined (i.e. *IP1* plus *IP1* or *VK1-NS* plus *VK1-NS*) the interactions are high. This is demonstrated in the bottom two columns of Figure 3 where the surface area of yellow fluorescence measured increases with over a time period of 36 hours. In the experiments with *E. invadens*-IP1 and *E. invadens-VK1-NS* combined in the same flask, the amebas dispersed evenly at 12 hours and 18 hours. After 36 hours not much yellow is detected [Figure 3, top two columns] demonstrating that when *E. invadens-IP1* and *VK1-NS* are combined they do not interact with physical contact as seen with like species interactions. Data was taken from three experimental trials with the best outcome. Three images were analyzed for each experimental trial and the data was averaged.

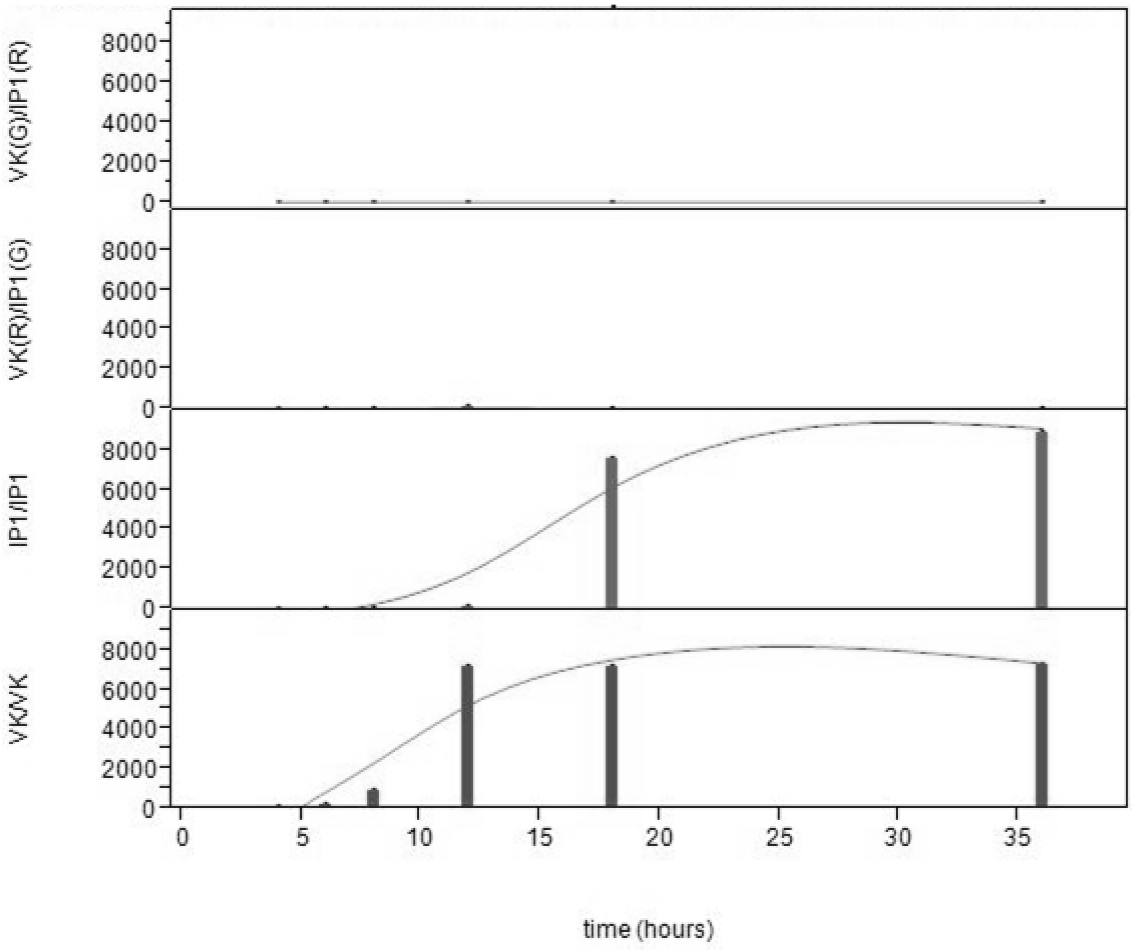


Figure 3: The mean surface area (microns^2) of overlapping red and green fluoresced *E. invadens- IP1* and *VK1-NS* representing combinatory interactions over time

When the yellow surface area is graphed for each combination it is clear that the interactions between like varieties increase over time [Figure 3]. When *E. invadens- VK1-NS* is observed there is a monotonic increase of interactions from 12 to 36 hours. The *E. invadens-IP1* has a larger slope between 12 to 18 hours but after 18 hours the slope decreases but the interactions continues to increase. When the different species are combined there is little to no interactions at 12, 18, or 36 hours [Figure 3].

Conditioned Media Experiments

In the filtered conditioned media experiments with *E. invadens-IP1*, a large number of interactions occurred. The surface area of the yellow was calculated and compared to the fresh media trials [Figure 4, top]. There is an increased surface area of interactions in the 12 hour conditioned media experiments [figure 4, center], and even more interactions with the 18 hour conditioned media [Figure 4, bottom]. This is also depicted in Figure 5a.

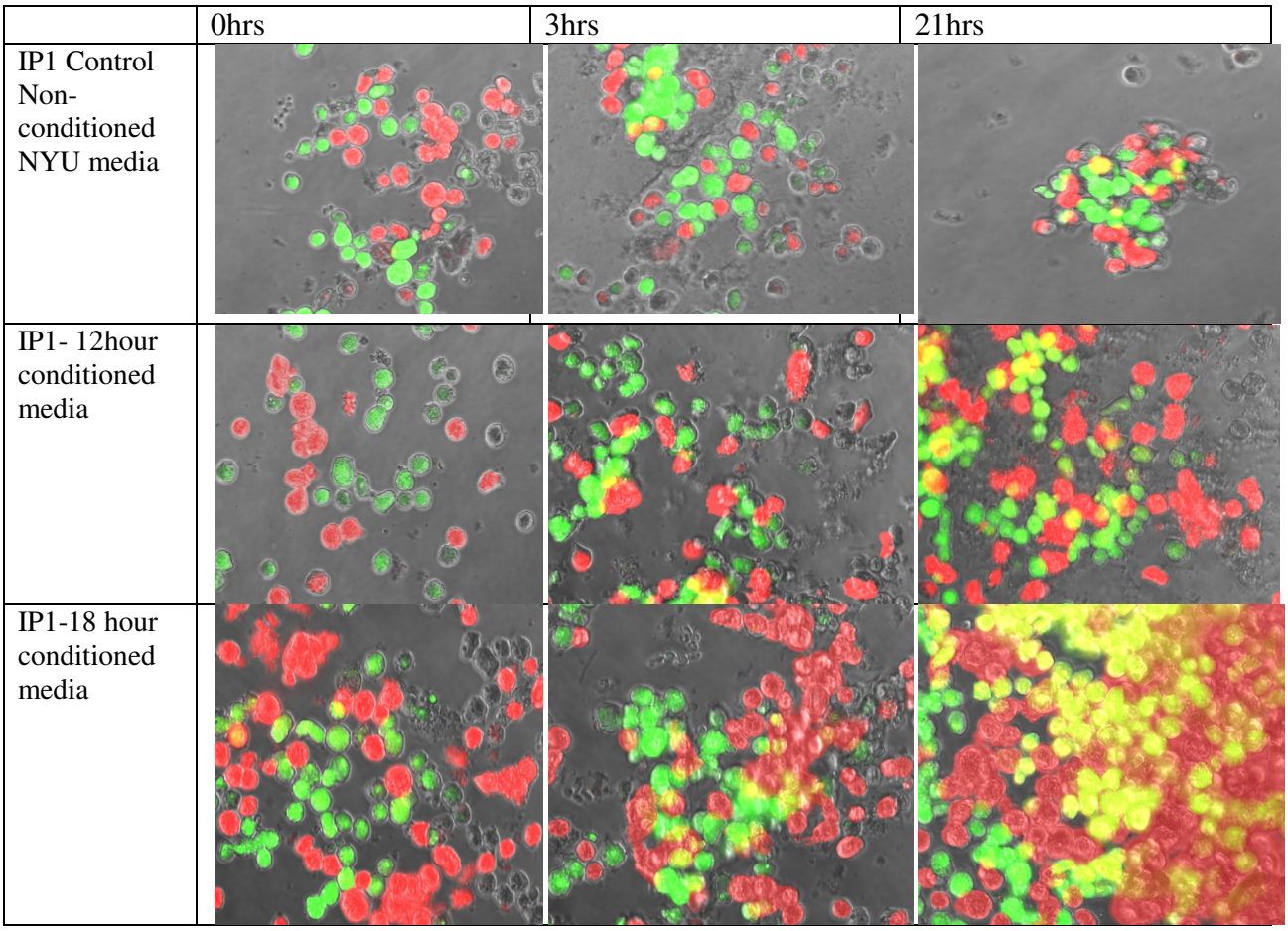


Figure 4: Fluoresced *E. invadens-IP1* in fresh NYU media (non-conditioned), 12 hour conditioned NYU, and 18 hour conditioned NYU over time [12, 18, and 36 hours].

When the filtered conditioned media was denatured, observed, and compared in the same sequence the surface area of yellow greatly decreases [Figure 5b]. When the two graph are compared the denatured media has less interactions over time compared to the filtered conditioned media that was not denatured [Figure 5 a,b].

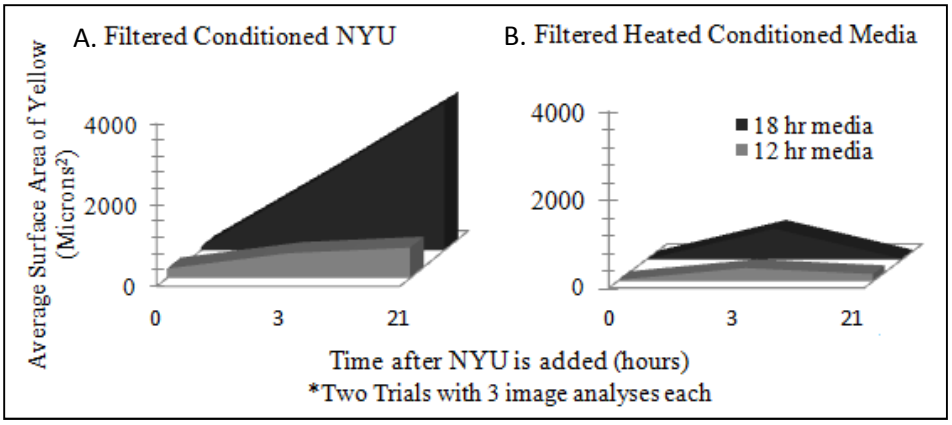


Figure 5a,b: Mean interactions (yellow surface area) of fluoresced *E. invadens-IP1* in 12 hour and 18 hour filtered conditioned media [a] and filtered heated conditioned media [b] in correlation with time.

SDS-PolyAcrylamide Gel Electrophoresis

When the conditioned media from both *E. invadens- IP1* and *VK1-NS* were ran through a SDS-PAGE gel electrophoresis there were obvious bands present in the both *E. invadens IP1* and *VK1-NS* conditioned medias (12, 24, 48 hrs) and no bands present in the control non-conditioned fresh NYU media [Figure 6]. Some bands are unique; *IP1* conditioned media exhibits some over expressed bands and some under expressed bands not present in the *VK1-NS* conditioned media, and vice versa [Figure 6].

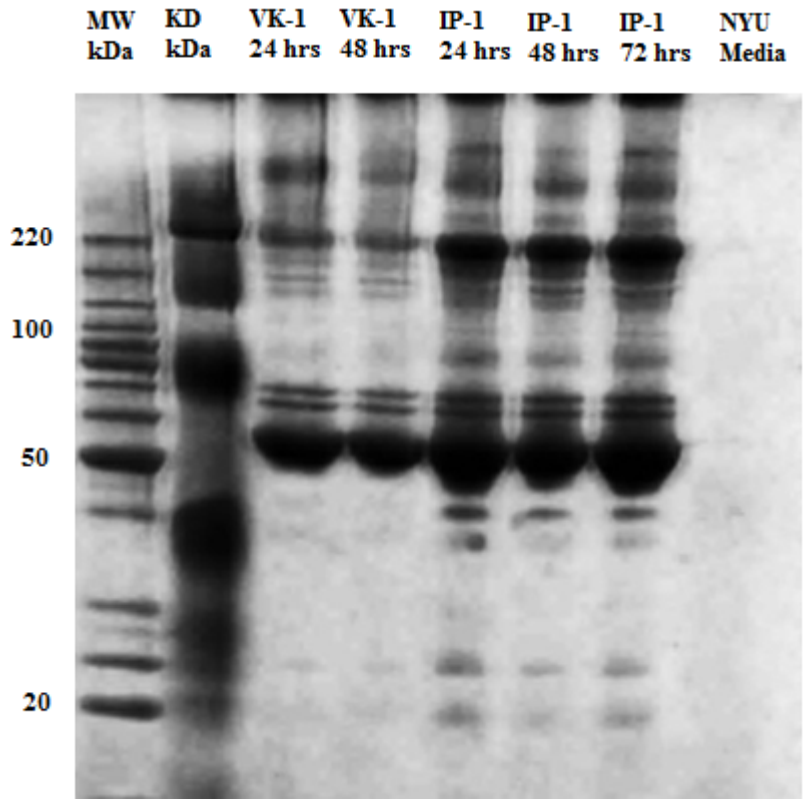


Figure 6: SDS-Page of 3x diluted conditioned media (*E. invadens- IP1* and *VK1-NS*) in comparison to control fresh NYU media.

Discussion and Conclusions

Recognition is a preliminary process in the evolution of cooperation in animals; however relevance in microorganisms and their behavior towards relatives has remained unclear. Our research of the *Entamoeba* lineage suggests that these protists discriminate and associate with like varieties, a feature previously attributed only to social microbes.

Mehdiabadi and colleagues utilized a social amoeba *Dictyostelium purpureum* to explore kin preference. In 2006, they revealed that, "the application of the kin-selection theory to microorganisms…provide further evidence that social microbes can show sophisticated behavior" (p. 881), using fluorescent micrograms taken with the same field of view at different

times. These fascinating results lead to the studies of recognition in non-social protozoan *Entamoeba invadens.*

Our results show that discrimination is an evolutionary adaptive trait for single-cell eukaryotes, such as *Entamoeba invadens*, as much as it is for cognitive species of animals. In assays in which *E. invadens IP1* and *VK1-NS* fluoresced, subspecies of differing varieties maintain distance from each other while similar varieties tend to interact with each other forming aggregates mediated by secreted molecules.

Ostrowski et al. suggest, "cells might avoid being victimized if they can discriminate among individuals and avoid those that are genetically different" (2008, p. 2376). We hypothesize that aggregation could play crucial role in the health of *Entamoeba* populations. *Entamoeba* trophozoites secrete chemical signals for aggregation to maintain a healthy number for survival (and reproduction). The aggregative behaviors, detailed in this study, might also have relevance for pathogenesis.

E. invadens IP1 and *E. invadens VK1-NS* are very similar strains but after analyzing quantitative values (average length and width) it is possible to conclude that *E. invaden IP1* is larger and shows differential aggregative patterns in comparison to *E. invadens VK1-NS.* Invitrogen CellTracker™ red CMTPX and Invitrogen Cell Tracer Vybrant® green CFDA SE fluorescent dyes do not have an effect on amoeba health or clustering in short term experiments. Negative controls demonstrate amoeba behavior without the effects of the dye. This reveals little difference between the amoeba fluorescently tagged and the amoeba not tagged allowing our conclusions to be drawn.

Measurable aggregation and behavioral cues in fluorescence micrographs of *Entamoeba* varieties reveal that trophozoites of each substrain aggregate only with the same variety suggesting they are able to associate based on behavioral and chemical communication.

Although a previous study has shown, "closely related but nonpathogenic *Entamoeba dispar* show no change in motility in response to conditioned medium implying that these responses are central to *E. histolytica* pathogenesis" (Zaki et al., 2006, p. 18751); Conversely, we have found that *Entamoeba invadens* aggregate at higher levels in conditioned medium in shorter periods of time.

Zaki et al. demonstrated that *E. histolytica* trophozoites secrete extracellular factors/signals that mediate *Entamoeba* chemotaxis (Zaki et al., 2006, p.49). Their research found that conditioned media enhanced the speed of random movement by more than 50% (Zaki et al., 2006, p.49), hinting that secreted molecules can modulate trophozoite movement. They speculate on the relevance of their findings for pathogenesis, however, their results are mostly qualitative.

Conditioned media assays demonstrate that *Entamoeba invadens* secrete a signaling protein(s) necessary for aggregation. These assays reveal that media that is conditioned for longer periods of time result in a higher mean interaction rate overall. Not only did the interactions increase in comparison to assays with fresh NYU media, but the interactions were seen at a shorter time period (after 3 hours as opposed to 18 hours).

When the conditioned media was denatured, observed, and compared in the same experiments the measured interactions greatly decreased, signifying that the denatured signaling

molecule was essential for aggregative behavior. These results are in the preliminary steps but could allude to a protein signaling mechanism seen in recognition assays.

Preliminary SDS-PAGE gel analysis of proteins secreted in the culture media (conditioned media) compared to media alone, demonstrate that *E. invadens VK1-NS* and *IP1* contain a number of unknown secreted proteins as indicated by the dark bands on the gel electrophoresis that are no present in the control. These bands also display some differences between the two subspecies samples. Some bands were distinct and seen over expressed or under expressed. These results could allude to variations between the two subspecies in their aggregative processes.

Ostrowski et al. (2008) propose, "genetic drift or adaptation to different components of the environment drives genetic divergence at the loci… [causing] discrimination…" (2008, p. 2367). These two subspecies may have differing signaling proteins/mechanisms leading to different recognition pathways. "Social interactions may thus depend on patterns of genetic diversity either across the genome or at key loci and a molecular population study of nucleotide variation can provide insights into the social dynamics within a species" (Flowers et al., 2010, p. 1). Flowers et al. reported that discrimination is directly correlated with genetic distance between strains in their experiments with amoeba *Dictyostelium discoideum* (2010, p.2). Also revealing that aggregation in *D. discoideum* is mediated by cAMP, a chemoattractant which triggers chemotactic movements of cells (2010, p. 1-2).

Overall, understanding the mechanisms of discrimination is just one step in understanding the development of both amebiasis and evolutionary pathways.

This study provides evidence that supports the research hypothesis that *E. invadens VK1-NS* and *IP1* discriminate and aggregate with like varieties, however more research is needed to confirm the exact mechanisms of these results. From this hypothesis two new experimental hypotheses emerged which need to be further explored. Either a secreted molecule mediates aggregation among members of the same group/variety or behavioral cues are based on chemical signaling.

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Appendices

Appendix A

Fluorescent Protocol for *E. invadens*: The following procedure is used to intracellularly fluoresce *E. invadens IP1* and *VK-NS* with Invitrogen red and green fluorescent dyes separately. Place tubes of amoeba cultures on ice for five to ten minutes (ex: 1 culture of VK-NS, 1 culture IP1) and immediately invert and tap the sides several times to scrap amoebas off the sides of the glass. Perform an accurate cell count using a hemocytometer of each culture tube used to ensure equal initial concentrations. Aliquot amoeba solution into 15 mL sterile plastic conical tubes (approx. 6ml each). Centrifuge at 4500 rpm for 20 minutes at 20°C to extract a pellet. The green dye is prepared using 1000 μL of Dimethyl Sulfoxide >99.9% (DMSO) to dilute the 50 μg Invitrogen Molecular Probes ® (Eugene, OR) Cell Tracer™ Green CFDA SE fluorescent protein. Prepare the red fluorescent dye using 300 μL DMSO to dilute the 50 μg Invitrogen Molecular Probes ® (Eugene, OR) CellTracker™ Red CMTPX fluorescent protein (We found these concentrations work best, however the concentrations can be adjusted with more or less DMSO). Remove the excess media and isolate the pellet. Dispense 100 μL of appropriate dye into each conical tube and resuspend. Incubate for 45 minutes at 23°C. Centrifuge at same conditions to achieve a pellet and remove the dye supernatant. Dispense 150 μL of fresh pre-warmed NYU media to each tube, resuspend and incubate at 37°C for 30 minutes. Centrifuge at same conditions to remove the media supernatant. Carefully add 500 μL of phosphate buffered solution without disrupting the pellet. After five minutes add 100 μL 3.7% formaldehyde to the pellet and let incubate at 23°C for 15 minutes. Dispense another 500 μL of phosphate buffered solution without disrupting the pellet and wait 5 minutes before removing the buffer supernatant leaving the pellet at the bottom. Add 500 μL of pre-warmed NYU to pellets and resuspend. Transfer the appropriate red and green fluoresced pellets into the same conical tube and mix. Aliquot 250 μL of amoeba mixture into a well [24 well-plates work best] adding appropriate amounts of filtered additive and fresh NYU media to the wells to achieve a 3:13 ratio. Cover and store at 23°C until observations are recorded at set intervals.

Conditioned Media Experimental Protocol: Once a culture tube reaches confluency perform a full media change (removing all waste and old media and replace with fresh filtered additive and media 3:13 ratio). After a set interval of time (12 hours and 18 hours for our experiments) extract the conditioned media from the culture tube and save in a clean sterile container (15 mL conical tubes work best). Filter the conditioned media through a 0.45 μm pore Nalgene filter via vacuum filtration. Store the filtered conditioned media at 4°C if needed for no longer than 48 hours. For long-term storage use a -20°C freezer. For accuracy, the fresher the conditioned media, the better. The filtered conditioned media was also denatured using a 90°C water bath the conditioned media was incubated for 30 minutes.

Growth Rate Protocol for *Entamoeba* Spp.: Culture tubes with maximum density at maximum confluency must be used. Remove media and add fresh media. Place culture tube on ice for 5-10 minutes and invert several times to loosen adherent cells from the glass. Perform an accurate initial cell count using the Nexcelom Vision Cellometer (Lawrence, MA) to accurately calculate the initial cell count for a 3 mL suspension. Add 3ml of this suspension to sterile glass culture tubes. Fill with the appropriate amount of media and filtered additive to achieve a 13:3 ratio. Use one tube ever 2 hours to perform an accurate cell count. The growth rate is calculated at each interval using the formula: $[(X_f - Xi)/X_i \bullet 100]$.

Appendix B

Table B1: NYU Media Protocol- (used in *E. invadens* and *E. histolytica*)

INGREDIENTs	grams
Potassium Phosphate, Dibasic	1
Potassium Phosphate, Monobasic	0.6
Sodium Chloride	1
Casein Digest Peptone	0
Tryptone	13
Yeast Extract	17
Neutralized Liver Digest	0
Glucose	10
L-cysteine Hydrochloride	1
Ascorbic Acid	0.2
Ferric Ammonium Citrate, brown form (22.8mg/mL)	0.0228
Final pH using 5M NaOH	6.8
Add DD2O (mL)	870
Final Volume (mL)	880

DD2O: double distilled H2O. Mix all ingredients; adjust the pH, and autoclave. Cool and filter with a 0.45µm pore Sterile Nalgene Cellulose Filter.

Table B2: Filtered Additive Protocol

INGREDIENTs	mL
Adult Bovine Serum	500
Penicillin-Streptomycin Solution	80
Vitamin Solution	150

Mix all ingredients, store at 4°C (last up to a month) or -20°C (for long term storage).

Table B3: Vitamin-Solution Protocol

INGREDIENTs	grams
Solution 1	

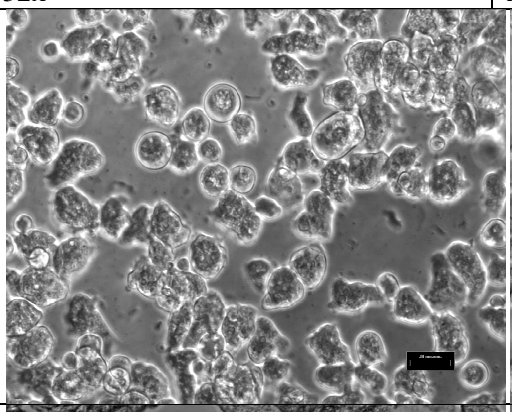
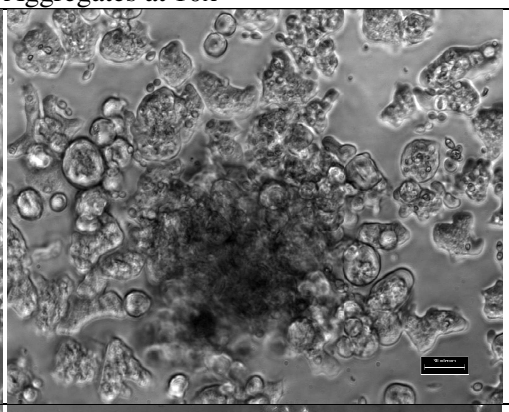
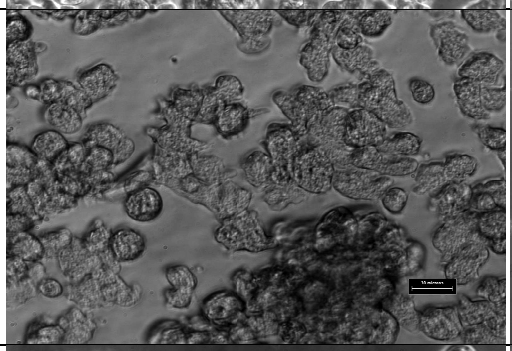
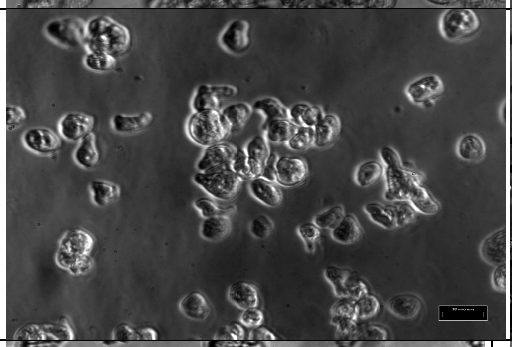
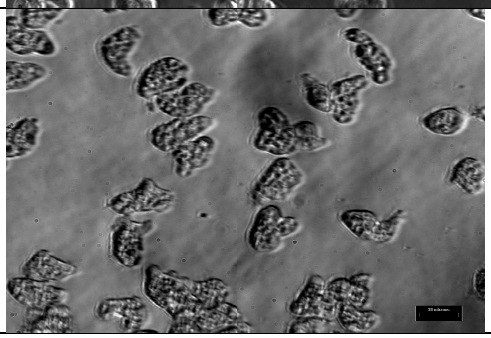
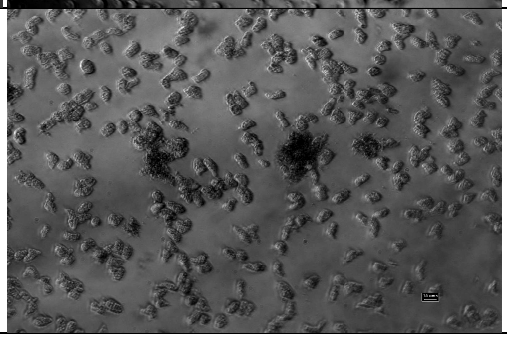
Niacinamide	.225
Pyridoxal Hydrochloride	.02
Calcium Pantothenate	.115
Thiamine Hydrochloride	.025
Vitamin B12	.006
Final Volume with DD2O (mL)	125mL
Solution 2	
Riboflavin	.035
0.1 M Sodium Hydroxide	minimum
Final Volume with DD2O (mL)	225mL
Solution 3	
Folic Acid	.0275
0.1 M Sodium Hydroxide	minimum
Final Volume with DD2O (mL)	225mL
Solution 4	
D-biotin	.01
Final Volume with DD2O (mL)	225mL
Solution 5	
D-L-6-8-Thiotic Acid (Oxidized Form) aka Lipoic Acid	.005
95% Ethanol (mL)	25
Tween-80	2.5
Final Volume with DD2O (mL)	150mL

DD2O: double distilled H2O. Combine solutions 1-4. Mix with solution 5. Bring final volume to 1000mL and filter the solution with a 0.45µm pore Sterile Nalgene Cellulose Filter. Aliquot and store covered (light sensitive) at 4°C

Table B4: Basic Morphology of *E. invadens*-IP1 and VK using ImagePro 6.3 Plus Software

	E. invadens-VK1-NS	***E. invadens-IP1***
Mean length (µm)	20-24	23-31
Mean width (µm)	15-20	20-24
Majority shape	elongated	rounded
Mean distance between single amoebas (µm)	23.41 µm	2.79 µm
Mean distance between clusters (µm)	69.71 µm	17.10 µm
Aggregation characteristics (avg. # amoeba in clusters)	Cluster in large groups >20	Cluster in large groups >20
Pathogenic in:	Varanus komodoensis	Serpentes
Incubation temperature	Room Temp (20°C)	Room Temp (20°C)
Aggregative behavior	IP1 clusters have greater distance	VK clusters remain closer together

Appendix C

Spp.	32x	Aggregates at 10x
E. invadens-VK		
E. invadens-IP1		
E. moskovski Larendo		
E. terrapinae		

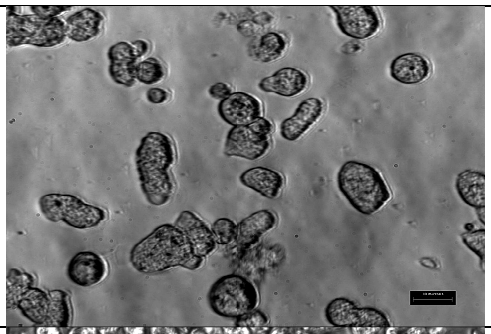
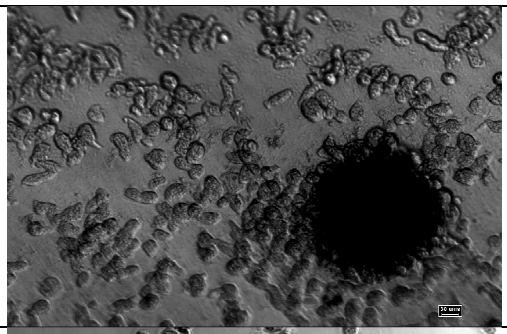
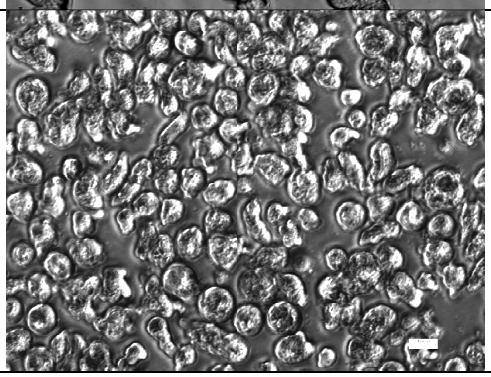
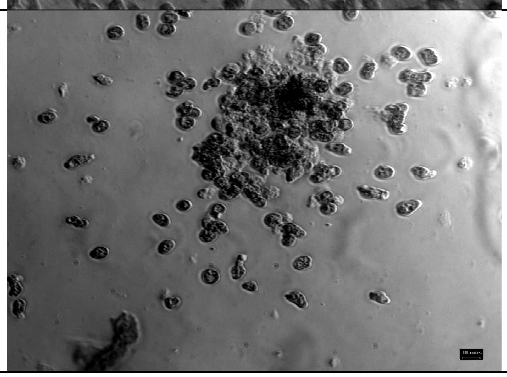
E. HM1 histolytica		
E. dispar (Images by George Perdrizet)		

Figure C1. Observations at 10x and 32x magnification of various *Entamoeba* species demonstrating the differences in aggregation, size, and shape

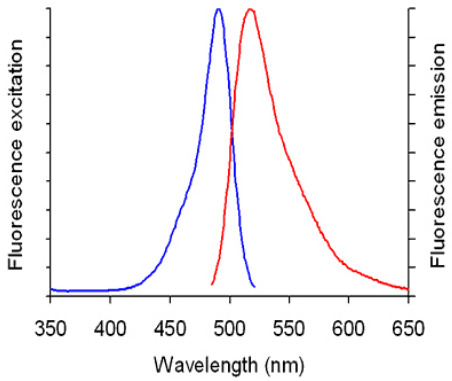
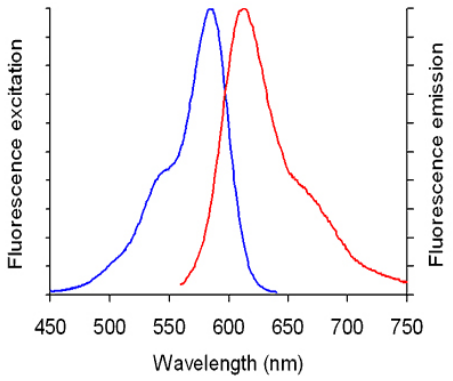
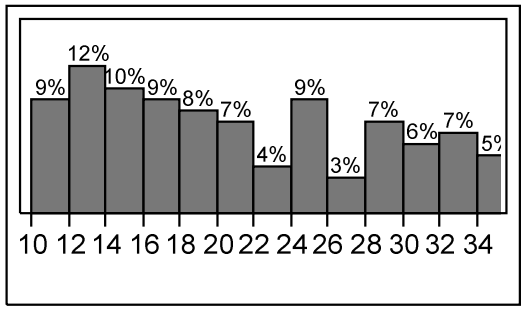
Cell Tracer™ CFDA Green	CellTracker™ CMTPX Red
	
Excitation-480nm Emission- 525nm	Excitation-580nm Emission-625nm

Figure C2: Invitrogen Molecular Probes® Cell Tracer™ CFDA Green and CellTracker™ CMTPX Red fluorescence excitation and emission graphed.

E. invadens IP1 cell average diameter distribution (microns)



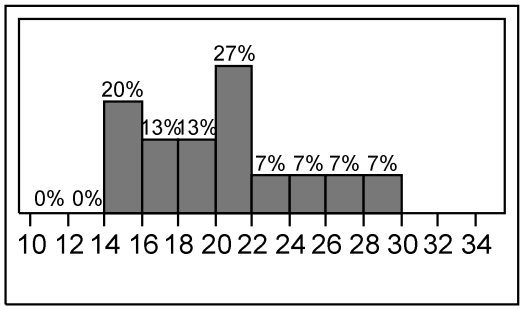
Quantiles

100.0%	maximum	36.91
99.5%		36.91
97.5%		36.265
90.0%		32.73
75.0%	quartile	28.82
50.0%	median	20.33
25.0%	quartile	14.56
10.0%		11.982
2.5%		10.78
0.5%		10.08
0.0%	minimum	10.08

Moments

Mean	21.579159
Std Dev	7.7785634
Std Err Mean	0.7519821
Upper 95% Mean	23.070037
Lower 95% Mean	20.088281
N	107
Sum Wgt	107
Sum	2308.97
Variance	60.506049
Skewness	0.3484872
Kurtosis	-1.168185
CV	36.046648
N Missing	0

E. invadens VK1-NS cell average diameter distribution (microns)



Quantiles

100.0%	maximum	28.07
99.5%		28.07
97.5%		28.07
90.0%		27.404
75.0%	quartile	23.38
50.0%	median	20.12
25.0%	quartile	16.34
10.0%		15.172
2.5%		15.13
0.5%		15.13
0.0%	minimum	15.13

Moments

Mean	20.206
Std Dev	4.2180324
Std Err Mean	1.0890913
Upper 95% Mean	22.541868
Lower 95% Mean	17.870132
N	15
Sum Wgt	15
Sum	303.09
Variance	17.791797
Skewness	0.5409743
Kurtosis	-0.687152
CV	20.875148
N Missing	92

Figure C3: *E. invadens-IP1* and *VK1-NS* individual cellular diameter distribution captured by Nexcelom Cellometer Vision and calculated using JMP Statistical Software.

Glossary

Aggregates Cells massed together into a dense cluster, usually consisting of more than twenty amoebas.

Altruism The practice of unselfish concern for the welfare of others or the unselfish sacrifice of one's self for the better of the community or population.

Amebiasis A gastrointestinal infection caused by *Entamoeba histolytica* that may or may not be symptomatic and can remain latent in an infected person for several years

Ciliates A single-celled protozoan of the phylum Ciliophora, distinguished by the possession of cilia or ciliary structures that reproduce asexually.

Chemoattractants Chemical substances causing adverse migratory affect.

Chemokines A large family of structurally small proteins that are released as a result of a stimulus. There are more than 50 different kinds of chemokines which bind to numerous receptors playing a crucial role in recruitment of various subsets.

Chemorepellents Chemical substances that cause a negative effect in motile cells. Chemorepellents can include inorganic salts, amino acids, or chemokines.

Chemotaxis Extracellular signals mediating motility of other cells in response.

Kin Recognition The ability for an organism to be able to identify and respond to close genetic relatedness and discriminate from non-kin not genetically close. A rapidly developing field in Evolutionary Biology.

Trophozoites The active feeding/growing stage of a protozoan's life-cycle, as distinct from its encysted stage.